Continuous cultivation of *Dehalococcoides mccartyi* with brominated tyrosine avoids toxic byproducts and gives tight reactor control

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Abstract

Dehalococcoides mccartyi strain CBDB1 is a strictly anaerobic organohalide-respiring bacterium with strong application potential to remediate aquifers and soils contaminated with halogenated aromatics. To date, cultivation of strain CBDB1 has mostly been done in bottles or fed-batch reactors. Challenges with such systems include low biomass yield and difficulties in controlling the growth conditions. Here, we report the cultivation of planktonic *D. mccartyi* strain CBDB1 in a continuous stirring tank reactor (CSTR) that led to high cell densities (~8×10^8 cells mL^{-1}) and dominance of strain CBDB1. The reactor culture received acetate, hydrogen, and the brominated amino acid D- or L-3,5-dibromotyrosine as substrates. Both D- and L-3,5-dibromotyrosine were utilized as respiratory electron acceptors and are promising for biomass production due to their decent solubility in water and the formation of a non-toxic debromination product, tyrosine. By monitoring headspace pressure decrease which is indicative of hydrogen consumption, the organohalide respiration rate was followed in real time. Proteomics analyses revealed that the reductive dehalogenase CbdbA238 was highly expressed with both D- and L-3,5-dibromotyrosine, while other reductive dehalogenases including those that were previously suggested to be constitutively expressed, were repressed. Denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA genes indicated that the majority of cells in the community belonged to the *Dehalococcoides* although the CSTR was operated under non-sterile conditions. Hence, tightly controlled CSTR cultivation of *Dehalococcoides* opens novel options to improve biomass production for bioaugmentation and for advanced biochemical studies.

Keywords: organohalide respiration; continuous stirred tank reactor; brominated phenolic compounds; online activity monitoring; bioremediation

1 Introduction

Bacteria of the genus *Dehalococcoides* are being commercially used to remediate aquifers contaminated with halogenated and highly persistent compounds, such as perchloroethene (PCE) (Steffan and Schaefer, 2016). Various *Dehalococcoides* strains have been isolated which are able to transform halogenated compounds including chlorinated dioxins, biphenyls (PCBs), benzenes, anilines, and thiophenes (Zinder, 2016). In addition, brominated and iodinated compounds such as brominated flame retardants and iodinated X-ray contrast media are reductively dehalogenated. This reductive dehalogenation is coupled in *Dehalococcoides* strains to energy conservation via a respiratory chain with hydrogen as an electron donor and acetate plus carbonate as the carbon sources. No other mode of energy conservation than this organohalide respiration has been detected in any of the isolated *Dehalococcoides* strain. On one hand, this high physiological specificity is good for *in situ* application because the occurrence of reductive dehalogenation can be tightly correlated with the presence of *Dehalococcoides*. On the other hand, the physiological specificity also makes the production of cell mass challenging: as a prerequisite, cell growth requires the presence of halogenated compounds which often have low water solubility and are often toxic at concentrations in the micromolar range. However, electron acceptors have to be added in the millimolar range to obtain sufficient cell numbers because of the molar growth yield of about 10^{13} - 10^{14} cells per mol of halogen substituent removed (Cooper et al., 2015). Such concentration of halogenated compounds is difficult to achieve in batch cultures and often causes growth inhibition. Another challenge in the cultivation of *Dehalococcoides* strains is the formation of less-halogenated compounds as end products of the dehalogenation reaction that can be more toxic than the parent compound. For example, many *Dehalococcoides* strains transform PCE only to the more toxic intermediate vinyl chloride (VC) which accumulates in the cultivation broth. A third challenge is the high oxygen sensitivity of *Dehalococcoides* strains that requires cultivation in gas-tight vessels with oxygen-protection by reducing agents. A last complication in the cultivation of *Dehalococcoides* strains is the monitoring of activity and cell growth, because no detectable turbidity can be observed during bacterial growth due to the small size of the cells, and offline monitoring of cell counts or compound concentrations is laborious. It is therefore time-consuming to determine the optimum harvesting moment when the bacteria reach their late exponential growth phase in a batch culture.

The genomes of all fully sequenced *Dehalococcoides* strains contain many different reductive dehalogenase operons, most of which encode a catalytic subunit RdhA and a small ~90 amino acid integral membrane protein RdhB which is assumed to be a membrane anchor for RdhA. In our previous work we have shown that the two proteins RdhA/RdhB form a distinct module which is part of a larger respiratory membrane-bound protein complex containing additionally a central module (three different subunits of a complex iron-sulfur molybdoenzyme, CISM, (Rothery et al., 2008)) and a hydrogen uptake module with a two-subunit, cytochrome-free hydrogenase HupL/HupS (Kublik et al., 2016; Seidel et al., 2018). RdhA and RdhB have shown to be inducible, and it is hypothesized that different RdhA/RdhB versions can be incorporated into the respiratory complex. The inducibility of the RdhA/RdhB module is supported by the almost invariable presence of the gene for a transcription regulator (two component systems or MarR-type regulators) upstream of the promotor of rdh-operons and has also been demonstrated by proteomic studies as a response to electron acceptor change (Franke et al., 2020; Yang et al., 2015). This emphasizes that for successful application of *Dehalococcoides* cultures in bioremediation not only the cell number and growth status are important but also the presence of appropriate RdhA/RdhB proteins.

In contrast to batch cultivation, the use of CSTR as bioreactor for cultivation allows to establish optimal cultivation conditions, by tightly controlling physicochemical parameters, such as pH,

redox potential or temperature. Cultivation in CSTR is also able to maintain the cell growth at a specific growth rate (i.e., specific doubling time). Cultivation of *Dehalococcoides* species in continuous or fed-batch reactors have been reported with PCE, trichloroethene (TCE), or VC as electron acceptors (Mao et al., 2019; Mortan et al., 2017; Berggren et al., 2013; Delgado et al., 2014; Richardson et al., 2002). Fermentable organic compounds such as lactate, pyruvate or methanol were used as carbon source and electron donor, and therefore led to enrichment of fermentative bacteria or methanogens and in many cases led to low abundance of *Dehalococcoides* species (Berggren et al., 2013; Mao et al., 2019; Mortan et al., 2017). By operating a continuous reactor at low hydraulic retention time (HRT, 3 d) and high TCE concentration (1-2 mM), Delgado et al. (2014) achieved high cell densities of *Dehalococcoides* ($>10^9$ cells mL^{-1}). In that study, lactate and methanol were fed and *Geobacter* as well as methanogenic archaea were detected. With high flow rate in the reactor, dominance of *Dehalococcoides* could be achieved. Concerns were raised on the accumulation of inhibitory volatile compounds (e.g., carbon monoxide) in the headspace in continuous reactors during the cultivation of *Dehalococcoides* (Mortan et al., 2017; Zhuang et al., 2014), but such inhibition was not observed in the study by Delgado et al. (2014).

In our study, we attempted to cultivate *Dehalococcoides* without fermentable substrates. The reactor medium contained acetate as an organic carbon source and hydrogen was added in the reactor headspace as the electron donor. Cultivation without fermentable substrates has two advantages: 1) it guarantees that *Dehalococcoides* will become the dominant species in the reactor since fermentative bacteria cannot thrive, 2) it enables online monitoring of dehalogenation activity through pressure drop caused by H_2 consumption. A novel electron acceptor, D- or L-3,5-dibrominated tyrosine, that can be reductively debrominated to the non-toxic debromination product, tyrosine, was introduced in this study. By setting up continuous cultivation in a CSTR, we aimed to assess the feasibility of a reliable and on-line monitored cultivation of *D. mccartyi* strain CBDB1, with acetate as the carbon source, and hydrogen in the headspace as the electron donor. Growth and dehalogenation were evaluated in regard to process stability and biomass yield. The presented reactor system has potential applications not only for the large-scale generation of highly active cell biomass of *D. mccartyi* for *in-situ* applications but also for the production of cell biomass for biochemical research.

2 Material and Methods

2.1 Synthetic medium used for cultivation

A synthetic medium was used for cultivation as influent of the semi-continuous stirred tank reactor. This medium was pH-buffered with 10 mM bicarbonate, reduced with 2 mM cysteine and amended with 5 mM acetate as a carbon source. It also contained a mixture of the vitamins biotin, thiamine, cyanocobinamide and dimethylbenzimidazole, apart from basic minerals and trace metals as described before (Adrian et al., 1998; Schipp et al., 2013). The synthetic medium was amended with D- or L-3,5-dibromotyrosine (abcr Chemie, Karlsruhe, Germany) depending on the type of cultivation performed. For cultivation in batch, starting concentrations between 0.5 and 4 mM of D-3,5-dibromotyrosine (D-DBT) were used; for setting up the CSTR, the synthetic medium used as influent was amended with 5 mM final concentration of D-DBT and later on, the reactor started operation using the synthetic medium amended with either 5 mM or 1 mM final concentration of L-3,5-dibromotyrosine (L-DBT) depending on the period of operation. The change from D-DBT to L-DBT was made due to the suitability of both isomers for the cultivation purposes and the much lower cost of L-DBT compared to D-DBT.

2.2 Reactor setup

The principles of the reactor setup have been described in a previous work for the cultivation of the anammox bacterium "*Candidatus* Kuenenia stuttgartiensis" (Ding et al., 2018). The reactor was gas-tight and contained sealed ports to connect a temperature probe and for adding medium and sampling through septa (Figure S1). Continuous operation of the reactor was enabled with an automated PSD/4 syringe pump (Hamilton, Bonaduz, Switzerland) equipped with a 5-mL syringe (model 1010.5 TLL with plunger made of ultra-high-molecular-weight polyethylene) and a six-port distribution valve (Hamilton, Bonaduz, Switzerland). By switching the valve to one of the six ports, the syringe pump could perform multiple alternative actions such as adding fresh medium / H_2 and removing reactor liquid. In programmed frequency as calculated based on the targeted hydraulic retention time (HRT), the syringe pump withdrew a fixed volume of reactor liquid (<2% of reactor volume) and added the same volume of fresh medium. The operation was controlled and monitored via a Raspberry Pi microprocessor running a self-developed Python script. The Raspberry Pi was also used to continuously monitor the gas pressure in the reactor headspace from a simple pressure sensor (MPX5100DP, NXP Semiconductors). In our study, two lab-scale continuous stirred tank reactors were used for cultivation. The first reactor had a volume of 1 liter and was used during Periods I and II of operation. pH values were regularly monitored offline by withdrawing samples for measurement, and adjustment of pH in the reactor liquid was done with 5 M NaOH. Throughout the reactor operation period, the pH was maintained at 6.8 ± 0.4.

2.3 Microbiological techniques

Samples for cell counting were withdrawn from the reactor with a sterile plastic syringe. When necessary, samples were diluted in MilliQ water before staining. Cells were quantified by direct cell counting on agarose-coated slides with an epifluorescence microscope after staining with SYBR-Green as described previously (Marco-Urrea et al., 2011). This method has a quantification limit of about 10^6 cells mL^{-1}.

Samples for DNA extraction were withdrawn from the reactor with a sterile plastic syringe and pelleted by centrifugation (twice at 14,000 g, 16 °C, 30 min). The resulting pellet was used for extraction of genomic DNA by using the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. PCR amplification of 16S rRNA genes was done using the GC-clamped forward primer 341FGC 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG CCT ACG GGA GGC WGC AG- 3' and the reverse primer 518R12 5'-WTT ACC GCG GCT GCT GG-3'. The PCR products were checked on 1% agarose gels and subjected to denaturing gradient gel electrophoresis (DGGE) using the DCode Universal Mutation Detection System (Bio-Rad). Gels were prepared at 10% polyacrylamide and a gradient of 20-80% denaturing agents, where 100% corresponds to 7 M urea and 32% v/v formamide. Amplified PCR products of 12 µL were loaded onto the gel and electrophoresis was run in 1× TAE buffer at 100 V and 60 °C for 16 h. The gel was SYBR-Gold stained, the bands were cut and DNA was eluted by overnight incubation in 10 mM Tris·HCl at pH 8.5. The eluted DNA was PCR amplified again (primers 341FGC-518R12), and the PCR products were purified and sent for Sanger sequencing (Eurofins Genomics Europe). The sequencing results were used for taxonomic identification by matching against the NCBI nt database using nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4 Ion chromatography, liquid chromatography, and shot-gun proteomics analyses

Samples from the reactor were periodically withdrawn for further analyses. The bromide ion concentration was quantified via ion chromatography using a Dionex-120 ion chromatograph equipped with an IonPac AS4A-SC (4 mm × 250 mm) column (detection limit: 10 µM).

Dibromotyrosine, bromotyrosine and tyrosine were analyzed by means of a high-performance liquid chromatography (HPLC) system with a LiChrospher 100 RP-18 (5 µm) LiChroCART 125-4 column with methanol and 0.1% formic acid as the mobile phase. Before HPLC analysis, primary amine groups in samples were derivatized using diethyl ethoxymethylenemalonate (DEEMM). To do this, 200 µL of sample was added into 1 mL of reaction buffer containing 7:3 v/v mixture of 50 mM borate buffer (pH 9) and methanol. Then 6 µL of DEEMM was added and the derivatization was carried out overnight at room temperature in the dark. The derivatized tyrosine and brominated tyrosines were detected in the HPLC at a wavelength of 280 nm (detection limit: 5 µM). For shot-gun proteomics, 1 mL of reactor liquid was centrifuged down at 14,000 g, 16 °C, 30 min to obtain the cell pellet. Cell disruption, trypsin digestion, and peptide desalting using C_{18} ziptips (Millipore, Merck) were done as previously described (Ding and Adrian, 2020). Desalted peptides were analyzed using nano-liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS) with an Orbitrap Fusion (Thermo), under the analytical conditions described before (Ding and Adrian, 2020). Mass spectra were evaluated with ProteomeDiscoverer software v2.4 (Thermo) and protein quantities were calculated by using the peak areas of precursor peptides in the MS1 scans using the Minora node of ProteomeDiscoverer. To calculate relative abundances (%), the abundance of a target protein was divided by the sum of abundances of all detected proteins in a sample.

2.5 Calculations

As shown in Equation 1, in a chemostat the dilution rate (D) is equal to the growth rate (µ) of the culture and the inverse of the hydraulic retention time (HRT). Therefore, the growth rate can be modified by changing the inflow rate (F) or the liquid volume of the reactor (V).

$$\mu = D = \frac{1}{HRT} = \frac{F}{V} \qquad \text{(Equation 1)}$$

The hydraulic loading rate was calculated as the molar amount of DBT entering the system per units of volume in the reactor per day of operation, and the specific loading rate was calculated as the molar amount of DBT entering the system per bacterial cell in the reactor per day of operation. These design parameters were calculated as follows:

$$Hydraulic\ Loading\ Rate\left(\frac{mmol\ DBT}{L \cdot day}\right) = \frac{[DBT]_{influent}\left(\frac{mmol}{L}\right) \times F\left(\frac{L}{day}\right)}{V(L)} \qquad \text{(Equation 2)}$$

where, $[DBT]_{influent}$ is the concentration of L-DBT in the influent (1 mM or 5 mM depending on the period of operation); F is the inflow rate applied in the CSTR and V is the liquid volume of the CSTR (1 L or 80 mL depending on the period of operation)

$$Specific\ Loading\ rate\left(\frac{mmol\ DBT}{cell \cdot day}\right) = \frac{Hydraulic\ Loading\ Rate\left(\frac{mmol\ DBT}{L \cdot day}\right)}{[cells]_{reactor}\left(\frac{cells}{L}\right)} \qquad \text{(Equation 3)}$$

where, $[cells]_{reactor}$ is the cell density of the culture in the CSTR

The molar growth yield was calculated considering the steady state operation as follows:

$$Molar\ Growth\ Yield\left(\frac{cell}{mol\ Br^-}\right) = \frac{[cells]_{effluent}\left(\frac{cells}{L}\right)}{[DBT]_{influent}\left(\frac{mol\ DBT}{L}\right) \times SR\left(\frac{mol\ Br^-}{mol\ DBT}\right)} \qquad \text{(Equation 4)}$$

where, $[cell]_{effluent}$ is the cell density of the effluent in the CSTR, which is equal to the cell density of the culture; $[DBT]_{influent}$ is the influent concentration of L-DBT (1 mM or 5 mM depending on the period of operation); SR is the stoichiometry ratio of the dehalogenation reaction of the dibromotyrosine, i.e., SR is equal to 2 mol bromide released per mol of dibromotyrosine dehalogenated.

3 Results

3.1 Debromination of 3,5-dibromotyrosine by *D. mccartyi* strain CBDB1

In preliminary experiments with batch cultures, it was shown that *D. mccartyi* strain CBDB1 can use D-DBT as a terminal electron acceptor and can grow on the basis of this reaction through anaerobic respiration with acetate plus carbonate as the carbon source and hydrogen as the electron donor (results not shown). Initial concentrations in the cultures between 0.5 and 4 mM of D-DBT were tested in batch cultures and growth was shown by direct cell counting, however complete inhibition of cell growth was observed with 4 mM of D-DBT. The maximum cell density obtained in these batch cultures was 1.0×10^8 cell mL^{-1}. The products of the D-DBT dehalogenation process were tyrosine and bromide, and the results matched the theoretical stoichiometry of 1:2 dibromotyrosine:bromide.

3.2 Start-up of continuous cultivation of strain CBDB1 in a CSTR

A pre-reactor was set up to produces active culture liquid for the inoculation of the main reactor. The pre-reactor vessel contained 950 mL cysteine-reduced medium with 1 mM D-DBT and was inoculated with 50 mL active culture containing 5.0×10^7 mL^{-1} *D. mccartyi* strain CBDB1 which was previously growing on 1 mM D-DBT in serum bottles. At day 0 of the pre-reactor operation, 6.7 kPa of H_2 were added into the headspace as the electron donor, and H_2 was regularly replenished when the pressure dropped. On day 11 of pre-reactor operation, cell density reached 1.7×10^7 cells mL^{-1} and the concentration of bromide ions (as determined by ion chromatography, see below) reached 1.6 mM. Then, the pre-reactor headspace was periodically flushed with H_2 and a small share of the pre-reactor liquid was periodically replaced with fresh medium containing 5 mM of D-DBT, with a maximum single dose of 50 mL (resulting in a concentration increase of 0.25 mM D-DBT in the pre-reactor). On day 21 of pre-reactor operation, the cell density reached 1.4×10^8 cell mL^{-1} and the bromide concentration reached 6.8 mM. Then, the pre-reactor was switched to a daily flow of 43.2 mL, equivalent to an HRT of 23 d. Such reactor flow was maintained for 23 days and 1 L of effluent was captured and stored at 4.5 °C. After one-month storage at 4.5 °C, the effluent was filtered through wide-pore filter paper under strictly anoxic conditions in the anaerobic tent to remove tyrosine crystals. The filtrate was transferred into the clean reactor vessel as the starting reactor liquid (day 0 of the main reactor). With the beginning of the main reactor operation, the electron acceptor was switched from 5 mM D-DBT to 5 mM L-DBT, while the HRT remained at 23 d.

On day 57, 16 mL of reactor liquid was mixed anoxically with 64 mL fresh medium containing no DBT as the starting reactor liquid for a downscaled 80-mL reactor. The reduction in reactor volume was decided in order to achieve higher daily flows in the reactor without stressing the syringe pump. For this 80-mL reactor, the concentration of L-DBT in the medium used as influent was lowered from 5 mM to 1 mM. Lowering the DBT concentration at the high flow rate was necessary because hydrogen transfer from headspace might have become limiting if the DBT concentration in the medium was too high. On day 60 (3 days after the 80-mL reactor was established), the continuous feeding of the CSTR was restarted.

3.3 Performance of the CSTR with a *D. mccartyi* strain CBDB1 enriched culture

The above-mentioned lab-scale CSTR (main reactor) was operated in continuous mode for 155 days with L-DBT as the electron acceptor. The operation was divided into six periods (I to VI) based on different hydraulic conditions applied to the reactor. The dilution rate was stepwise increased to achieve fast growth. Thus, the HRT was decreased from 23 days in Period I to 4 days in Period VI (Figure 1). Successful continuous reactor operation was achieved without accumulation of brominated compounds and the results again accorded to the theoretical molar stoichiometry of 1:2 dibromotyrosine:bromide ions. Decrease of reactor headspace pressure was constantly observed, indicating H_2 consumption.

Figure 1 shows the L-DBT loading rate and the L-DBT specific removal activity achieved for each period of operation. The specific removal activity was not plotted for Period I because the reactor had not reached the pseudo steady state yet. Still, the measured values of L-DBT concentration were very low during this period. In fact, the concentration of L-DBT in the reactor was under the detection limit (5 μM) during most of the operation time. The same was true for the potential dehalogenation intermediate 3-monobromotyrosine. Accumulation of L-DBT was only observed during the start-up of the continuous operation (Period I), with 0.03, 0.02, 1.3 and 0.02 mM of L-DBT measured on days 1, 8, 17 and 28, respectively, indicating that the culture had to adapt to the conditions during Period I. The highest L-DBT concentration was detected on day 17 which was due to the restart of the operation after one day of starving conditions (fresh medium input was stopped for one day). For the rest of the operation, we did not again observe a significant increase of L-DBT in the reactor, indicating that the bacterial growth was continuously limited by the concentration of L-DBT. From Period II to IV, the removal rate was maintained at an average value of $7\times10^{-12} \pm 3\times10^{-12}$ nkat cell^{-1}. It is important to notice that, when the hydraulic loading rate decreased from Period II to III, the specific loading rate (i.e., the amount of L-DBT loaded per cell) was still maintained. This is due to the fact that chemostat operations allow for uncoupling of the growth rate and the cell density of the culture, and here both parameters changed when moving from Period II to III (higher μ and lower electron acceptor concentration in the influent were applied, resulting in an unchanged specific loading rate). In Periods V and VI, the L-DBT specific loading rate was increased and no increase of the L-DBT concentration was observed in the reactor liquid. The average specific removal rate achieved during Period V and beginning of Period VI was $2.5\times10^{-11} \pm 0.5\times10^{-11}$ nkat cell^{-1}. On day 149, the L-DBT loading rate was further increased to 6.1×10^{-12} mmol DBT cell^{-1} day^{-1} and again no accumulation of brominated compounds occurred.

During Periods I and II, the formation of whitish turbidity was observed in the reactor, but the operation was not affected. Under these circumstances, when the effluent of the reactor was collected and stored at 4.5 °C, the formation of needle-like crystals occurred, while the supernatant was clear (Figure S2). Since the tyrosine concentration in the CSTR (5 mM) was higher than the tyrosine solubility in water (2.5 mM), the precipitates were expected to be tyrosine crystals, which was the final product of the debromination process from L-DBT. The identity of the crystals was confirmed with HPLC-UV by comparing to the retention time and absorbance spectra of a neat tyrosine standard.

The continuous cultivation in the CSTR allowed for the calculation of H_2 consumption based on the decrease of pressure in the reactor headspace. Periodically, the actual gas consumption was calculated from the pressure data and it matched with the theoretical values expected from the molar stoichiometry of 1:2 dibromotyrosine:hydrogen (Figure 2, Table 1). Hence, the extent of the dehalogenation process of L-DBT was assessed in real-time during the entire operation of the CSTR. For instance, on day 17, when the medium feeding was stopped, no decrease in headspace pressure was observed (i.e., no consumption of H_2).

The cell density obtained in the CSTR varied according to the operation conditions (Figure 3) and the maximum cell density achieved was of 8.5×10^{8} cell mL^{-1}, only limited by the L-DBT concentration. The decrease of cell density observed from Period II to III was related to the decrease of L-DBT concentration in the influent from 5 mM to 1 mM, and the decrease of cell density observed from Period IV to V was due to a culture withdrawn intentionally, performed during sampling.

The molar growth yield of the culture growing on L-DBT varied during the different periods of cultivation, resulting in $9\times10^{13} \pm 5\times10^{13}$ cell mol^{-1} Br^{-}. The highest molar growth yield was obtained on Periods III and IV, with an average value of $1.2\times10^{14} \pm 0.3\times10^{14}$ cell mol^{-1} Br^{-}. Under the same conditions of electron acceptor influent concentration (Periods III to VI, 1 mM of 3,5-DBT), the higher the growth rate imposed (i.e., the younger the culture), the lower the molar growth yield observed (Figure 4).

Figure 5 shows an image of the SYBR green stained cells withdrawn from the CSTR and the results of one of the DGGE analyses performed on a culture sample. On one hand, during the entire operation the typical spherical cell shape of *D. mccartyi* strain CBDB1 was observed in samples directly withdrawn from the CSTR. On the other hand, DGGE results demonstrated that a high enrichment in *D. mccartyi* strain CBDB1 was maintained when operating the CSTR, despite the reactor was not operated under sterile conditions.

3.4 Expression of reductive dehalogenases

Samples of the CSTR culture were periodically withdrawn to evaluate the expression of reductive dehalogenases. Shotgun proteomics analyses were performed with samples of days 8, 21, 57, 112 and 144, all from culture growing on L-DBT. A sample from the culture growing on D-DBT in the pre-reactor (see Section 2.2 for details) was also used for shotgun proteomics for comparison. A total of nine different RdhA proteins were identified to be expressed, although not in all the periods of operation (Table 2, Table S1). The relative abundance of the different RdhA also varied among periods. CbdbA84, CbdbA238 and CbdbA1092 were identified in all the samples, although no correlation with time of operation and relative abundance was observed. Overall, CbdbA238 and CbdbA1092 showed the highest abundance compared to the other RdhA identified in the samples, with the exception of sample from Period IV, when CbdbA84 was also highly expressed. Details on the protein abundances can be found in the Supplementary Material.

A cross-comparison of RdhA abundance among CBDB1 culture fed with different halogenated compounds (Table 2) showed an association of expressed RdhA with the type of halogenated compound added: CbdbA80 and CbdbA84 were highly expressed when halogenated benzenes were provided, whereas CbdbA1092 was only detected when the halogenated compound contained a phenol ring (DBT, tetrabromobisphenol A, or bromophenol blue). The expression of CbdbA238 was exclusively associated with culture fed with L- or D-DBT, indicating specific induction by this compound. A significant difference between the induction by L- and D-DBT was not found.

4 Discussion

4.1 CSTR as a strategy for cultivating *Dehalococcoides*

The results of this study show how *D. mccartyi* strain CBDB1 can be cultivated in continuous mode in a CSTR using L-DBT as electron acceptor, hydrogen as electron donor and acetate as carbon source. The CSTR design used here was introduced in a previous work of the authors as a very useful tool to cultivate anammox bacteria (Ding et al., 2018), but it was not tested so

far for anaerobic cultivation where a substrate was added from the gaseous phase. This is an important issue when cultivating the strictly anaerobic bacteria *D. mccartyi* strain CBDB1 because potential negative headspace pressure could lead to air leakage, which would trigger the cessation of the dehalogenation process. The gas-sealed setup, the continuous monitoring of pressure in the headspace and the automated maintenance of a positive pressure in the gas phase allowed for a successful long-term stable operation of the CSTR, demonstrating the feasibility of using the CSTR for cultivation under anoxic conditions. Besides, the continuous monitoring of headspace pressure allowed the quantitative evaluation of the dehalogenation process in real-time because the rate of pressure decrease quantitatively correlated with the L-DBT consumption rate. Such precise online monitoring is key for controlling the dehalogenation process in continuous mode in CSTR. In addition, the CSTR operation provided robustness to face changing loading rates. As shown in Figure 1, L-DBT concentration in the CSTR was zero even when the specific loading rate was increased from day 120 onwards. The increase in removal rate without any accumulation of L-DBT indicated that the culture growth was limited by a lack of the halogenated electron acceptor. Hence, the *D. mccartyi* strain CBDB1 culture was not yet at its maximum dehalogenation capacity. In general, cultivation in continuous mode can achieve higher dehalogenating rates than in batch mode, because the continuous mode maintains the bacteria in their exponential growth phase. This is best illustrated by the accumulation of dibromotyrosine on day 17 after starving the reactor for only one day (Figure 1), showing cells that have been starved for only one day were not able to cope with the loading rate that was applied before. The dehalogenation rates obtained in this study are in the same range of those reported by Delgado et al. (2014) for dichloroethene dehalogenation in a CSTR containing a *Dehalococcoides* dechlorinating mixed-culture (0.5-1.4 mmol Cl^- L^{-1} d^{-1}).

Another important advantage of the CSTR design is the possibility to control the cell density of the culture by increasing the substrate concentration in the influent (Madigan et al., 2006). Figure 3 shows that the highest cell density in the CSTR was obtained when the influent concentration was 5 mM L-DBT. As expected in a chemostat, the higher the electron acceptor concentration in the influent the higher the cell density of the culture. In this period, the steady state was probably not achieved due to the high HRT applied (HRT of 23 and 17 days in Periods I and II, respectively), and thus the maximum cell density was not achieved. Higher cell numbers would be expected if there were no limitations in growth by lack of electron acceptor and/or if higher influent concentrations than 5 mM were used. In this sense, the cultivation in a CSTR allows the use of higher concentrations of electron acceptor than the ones used in batch systems, because of the immediate dilution of influent when entering the reactor, overcoming the potential toxicity or inhibition by electron acceptor addition. We observed complete inhibition of the growth of *D. mccartyi* strain CBDB1 in batch cultures when using initial concentration of 4 mM D-DBT, whereas 5 mM inflow concentration generated no problem in the CSTR of this study. Despite the observed electron acceptor limitations, the bacterial growth was sustained in the long-term in our CSTR, even when imposing a high inflow rate in the system with a doubling time of 3 days in Period VI (*D. mccartyi* strain CBDB1 was reported to have a doubling time between 1 and 3 days (Löffler et al., 2013)). Also, the maximum cell density achieved in the CSTR cultivation (8.5×10^8 cell mL^{-1}) was higher than the usually achieved maximum cell number in batch cultures ($1–5 \times 10^8$ cells mL^{-1} in a fully-grown culture). The feasibility of obtaining high cell density *D. mccartyi* strain CBDB1 cultures in continuous mode makes the CSTR configuration a promising choice for establishing bioremediation strategies for contaminated sites where a continuous feeding of high cell number cultures with high activity are needed.

4.2 3,5-dibromotyrosine as suitable electron acceptor for *D. mccartyi* strain CBDB1

The cultivation of *D. mccartyi* strain CBDB1 using 3,5-dibromotyrosine was introduced here for the first time and it was demonstrated that strain CBDB1 grows with both isomers D- and L- of dibromotyrosine as electron acceptors. The brominated version of the amino acid tyrosine was selected on the basis of its presumed harmlessness and its decent water solubility (10 mM at room temperature), so its use for routine cultivation was considered to be picture-perfect: the final product of its dehalogenation is non-toxic and does not contain any halogen substituent anymore, so that the waste disposal is easy to handle in the laboratory. Altogether, this organohalide is presented as promising electron acceptor to be used for cultivation purposes of *D. mccartyi* strain CBDB1. In this study, growth of *D. mccartyi* strain CBDB1 was shown when using both 5 mM and 1 mM of L-DBT, which represent very high concentrations in the haloaromatics dehalogenation field. In comparison, with other brominated organic compounds, concentrations in the micromolar range have often to be used to avoid toxicity and to observe growth (Wagner et al., 2012; Yang et al., 2015). Hence, the possibility of using concentrations as high as 5 mM is beneficial in terms of achieving high biomass concentrations for bioremediation purposes. The potential limitation of the use of high concentrations of 3,5-DBT as electron acceptor was the formation of precipitates of the final product, tyrosine, which could trigger mass transfer problems in the reactor. This issue is addressed in the Section 4.3 below.

Full debromination of 3,5-DBT to the non-halogenated product and the associated growth of strain CBDB1 was observed. The obtained molar growth yield ($9\times10^{13} \pm 5\times10^{13}$ cell mol^{-1} Br) was in the range of molar growth yields reported for *D. mccartyi* strain CBDB1 growing with other brominated or chlorinated electron acceptors, such as bromobenzenes (1.8 to 2.8×10^{14} cell mol^{-1} Br (Wagner et al., 2012)), bromophenols (3×10^{14} (Yang et al., 2015)), chlorobenzenes (7×10^{13} to 1×10^{14} cell mol^{-1} Cl (Jayachandran et al., 2003)) or chlorophenols (8×10^{13} cell mol^{-1} Cl (Adrian et al., 2007a)). All these reported molar growth yields were determined in batch cultures and, thus, the molar growth yield was an integrated value over the whole incubation period including all the different growth phases of batch cultivation. Here, we induced different stable growth rates (cell doubling times) by changing the inflow rate and found that the molar growth yield was lower when the growth rate was increased, i.e., when doubling time decreased (Figure 4). Such decrease in molar growth yields at high growth rate is counterintuitive, as less maintenance energy is spent when cells grow faster and should have led to higher molar growth yields. Multiple reasons could have caused such a phenomenon: 1) it could be that with increased inflow rate, cells detected higher availability of halogenated compounds and expressed more respiratory proteins (Table S1), leading to more energy spent on each cell division; 2) generally higher protein and DNA content and larger cell size in fast-growing cells; 3) biofilm may have accumulated on the reactor vessel, leading to lower cell molar growth yields as calculated based on numbers of planktonic cells in reactor liquid, although we did not observe visible biofilm formation. However, the maintenance energy requirements seemed to play an inferior role. Further investigation is needed to elucidate the possible causes.

L- and D-DBT are not obvious natural substrates widely present in the environment to support growth of *Dehalococcoides* species, but can still be respired by strain CBDB1. Similar phenomena have been observed with other halogenated compounds that are used by strain CBDB1 for anaerobic respiration, e.g., with bromophenol blue. Also, strain CBDB1 did not encounter any brominated tyrosine since its transfer to continuous lab cultivation in 1995 and there was no difference in the growth between the two DBT isomers. This all supports previous conclusion that the reductive dehalogenases must react relatively unspecific towards a range of halogenated aromatics (Cooper et al., 2015). Evidence accumulates that the side groups but not the halogen substituents determine the specificity of reductive dehalogenases in *Dehalococcoides*.

The stoichiometric conversion of hydrogen and DBT to tyrosine and the absence of any other electron acceptors or fermentable substrates demonstrated that strain CBDB1 was using DBT as a respiratory electron acceptor. The specific expression of RdhA proteins in cells withdrawn from the CSTR indicated their involvement in this process. *D. mccartyi* strain CBDB1 contains 32 different reductive dehalogenase operons on the genome, each constituted by one *rdhA* and one *rdhB* gene (plus regulatory genes) which encode the catalytic enzyme RdhA and its putative membrane anchor RdhB. However, so far no clear correlation was established between the transcription of these *rdhA gene*s and the exposure to particular organohalides, and it was suggested that a low and steady transcription of most of them occurs (Maillard and Willemin, 2019). Our results contribute to this idea since up to 9 different RdhA proteins were identified but only two proteins were present with high abundances in all the samples collected from the reactor. This fact suggests that those two proteins actually catalyzed the debromination of dibromotyrosine. On one hand, we observed the specific induction of the RdhA protein CbdbA238 which was not identified with previous halogenated electron acceptors, suggesting specificity for reduction of dibromotyrosine. On the other hand, the high expression of CbdbA1092 supports the hypothesis that this protein could be involved in the dehalogenation of brominated oligocyclic phenolic compounds, as previously suggested by Yang et al. (2015) when cultivating strain CBDB1 with bromophenol blue as electron acceptor. In fact, unpublished results from our lab showed that CbdbA1092 was the only RdhA identified in batch cultures when cultivating strain CBDB1 on bromophenol blue after five passages to fresh medium (Table 2).

The RdhA CbdbA84, often identified in cultures of strain CBDB1, was identified in this study in all the samples, although with significant abundance only at the end of the operation. This RdhA was initially associated with the dehalogenation of chlorinated benzenes (Adrian et al., 2007b), and it was also identified in the presence of brominated benzenes (Wagner et al., 2012). It is worth to notice the disappearance of the reductive dehalogenase CbdbA80 commonly found in protein samples of strain CBDB1 cultures, which could indicate that this RdhA is not broadly expressed as it was thought and, more specifically, CbdbA80 is not specific for dehalogenation of dibromotyrosine.

4.3 Practical implications for the design of bioremediation strategies

The stable and easily-maintainable operation, the resilience to face high loading rates, the possibility of controlling cell density and maintaining the exponential phase of growth, and the capacity of overcoming electron acceptor toxicity issues, make the CSTR design a promising tool for producing cells of *D. mccartyi* strain CBDB1 for downstream applications. The CSTR allows the control of the growth rate and the cell number independently. For producing biomass at a maximum and stable cell density for full-scale applications of bioremediation, it will be of paramount importance to determine (i) the optimum growth rate of the culture under the reactor conditions and (ii) the optimum influent concentration of the electron acceptor that can be handled. In addition, the success on growing an active culture using 3,5-dibromotyrosine that produces non-toxic tyrosine makes the tandem CSTR-DBT the perfect choice to produce high biomass concentrations of *D. mccartyi* strain CBDB1 for downstream processes, either bioremediation processes or research experiments. The only potential drawback of using 3,5-dibromotyrosine as electron acceptor is the relatively low water solubility of the end product of the dehalogenation, tyrosine, since at concentrations higher than 2 mM it can already trigger the formation of precipitates in the reactor. This precipitate formation could be a suitable surface for biofilm formation and aggregations of bacteria could lead to mass transfer limitations which limit the *D. mccartyi* strain CBDB1 growth. However, in this study such limitations were not observed despite of the formation of precipitates during Periods I and II. On the contrary, the precipitation of tyrosine allowed for an easy separation of it, and thus its

removal from the effluent by filtration. Hence, the precipitation of tyrosine could also be seen as beneficial to obtain a clean effluent. Also, tyrosine is a natural compound that, although it is not incorporated into the metabolism from external sources by strain CBDB1 (Marco-Urrea et al., 2012) it can be used by many bacteria as carbon or nitrogen source and thus it should not represent a contaminant for *in situ* applications, so that the effluent of the reactor does not need any additional cleaning step before application for bioaugmentation.

Due to the substrate-specificity of reductive dehalogenases, sometimes it is inevitable to use organohalides that are toxic or organohalides that produce toxic dehalogenated products. In such cases, continuous cultivation of *Dehalococcoides* is still feasible with our reactor design, as long as necessary precautions are taken. The pressure monitoring could even be applied when volatile organohalides or products are involved, e.g., dechlorination from TCE to ethene. In such cases, an adjustment to the H_2 consumption rate has to be made to compensate for partial pressure increase that is caused by the production of the volatile compound (e.g., ethene). In summary, the reactor setup presented in this study is broadly applicable to other *Dehalococcoides* or *Dehalogenimonas* strains with organohalides other than brominated tyrosines.

5 Conclusions

D. mccartyi strain CBDB1 was successfully cultivated in continuous mode in a semi-continuous stirred tank reactor and long-term stable operation was achieved. Acetate, instead of fermentable organics, was used as the carbon source. The simple and easy-handled design, the feasibility of monitoring the dehalogenation process in real time, the reactor resilience to confront high organohalide loading rates and the possibility of obtaining high cell density cultures, make the CSTR a promising tool for stable cultivation.

Growth on the compound 3,5-dibromotyrosine which produces non-toxic tyrosine was demonstrated and molar cell yields obtained were in the same range of those previously shown for *D. mccartyi* strain CBDB1. The specific reductive dehalogenases CbdbA238 and CbdbA1092 were expressed when using dibromotyrosine as electron acceptor.

The tandem CSTR-dibromotyrosine is a good choice for producing high volume of *D. mccartyi* strain CBDB1 cultures at high biomass concentration, which can be used in downstream processes, either for establishing bioremediation strategies in contaminated sites or for their further use in research.

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Figure Legends

Figure 1. Performance of the CSTR during the different periods of operation in regard to the L-3,5-dibromotyrosine (DBT) loading rate and the specific removal rate achieved. The concentration of L-DBT in the CSTR is also presented. Period I and II correspond to reactor operations using an influent with 5 mM of L-DBT and 1 liter of reactor volume. Period III to VI correspond to reactor operations using an influent with 1 mM of L-DBT and 80 mL of reactor volume. Units in nkat per cell refer to nmol of dibromotyrosine removed per second per cell.

Figure 2. Reactor headspace pressure change (in gas volume equivalent) during a three-day period (day 27-day 29 of the pre-reactor) of continuous operation using medium containing 5 mM D-DBT. Pressure increase at 29 h and 53 h was due to addition of 10 mL H_2 into the headspace. The periodical short-term drop and rise in pressure were due to the regular operation of the pump replacing 5 mL of culture liquid with 5 mL of fresh medium.

Figure 3. Cell density in the CSTR during the different periods of operation.

Figure 4. Average molar growth yield calculated for Periods III and IV (doubling time of 8 and 6 days, respectively) and Periods V and VI (doubling time of 4 and 3 days, respectively). ** denote p value < 0.01 in a t-test.

Figure 5. A) Epifluorescence microscopic image of the cells from the CSTR culture after SYBR Green-staining; B) DGGE profile of bacterial DNA extracted from the CSTR culture (lanes 1 and 5) and from three different pure or mixed batch cultures of our lab (lanes 2, 3 and 4). Lane 1 and 5 correspond to 2 months and 3 months operation time of the reactor, respectively, on L-DBT. Cut and sequenced bands are indicated by black triangles; C) Sequencing results of selected DGGE bands from the DGGE profiles. (* denote that sequences did not show a significant similarity in BLAST; ** denote that the sequence retrieved from the sequencing process was of poor quality).

Fig. 1

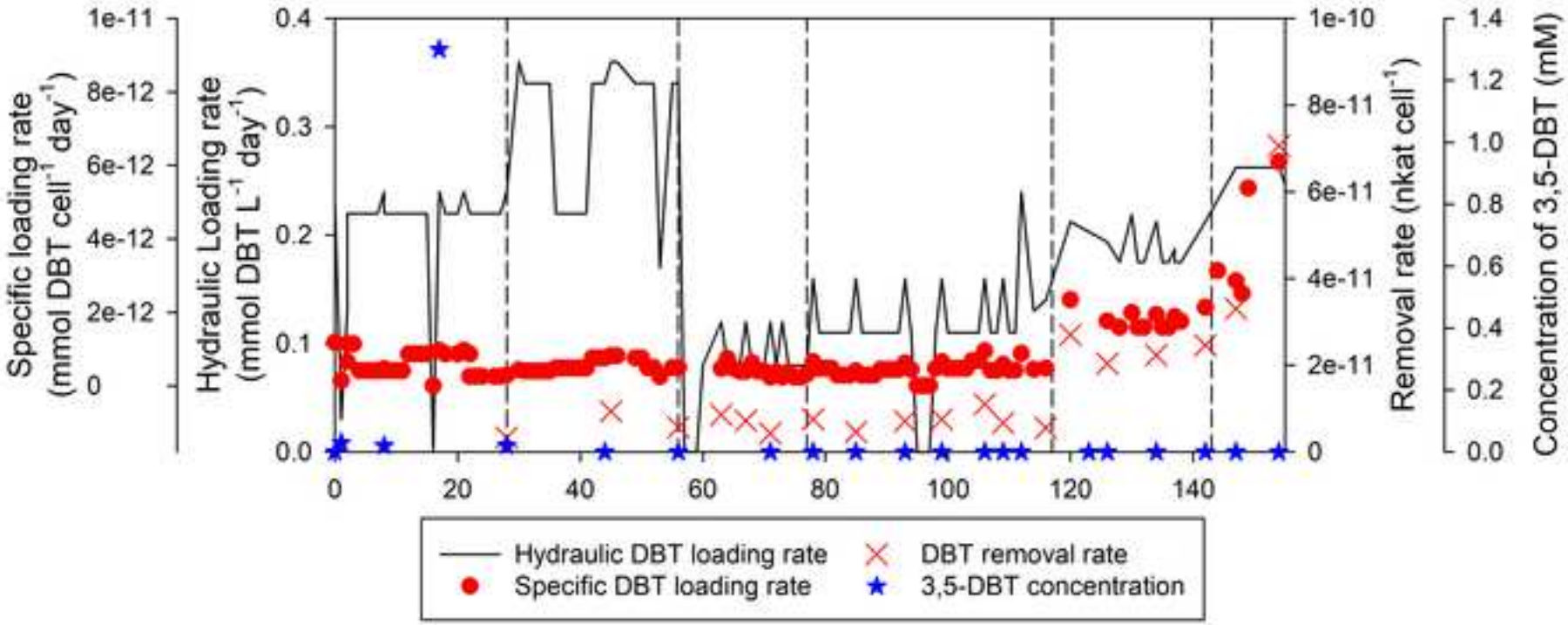

Specific loading rate (mmol DBT cell^{-1} day^{-1})
Hydraulic Loading rate (mmol DBT L^{-1} day^{-1})
Removal rate (nkat cell^{-1})
Concentration of 3,5-DBT (mM)
1e-11
8e-12
6e-12
4e-12
2e-12
0
0.4
0.3
0.2
0.1
0.0
1e-10
8e-11
6e-11
4e-11
2e-11
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
Hydraulic DBT loading rate
Specific DBT loading rate
DBT removal rate
3,5-DBT concentration

Fig. 2

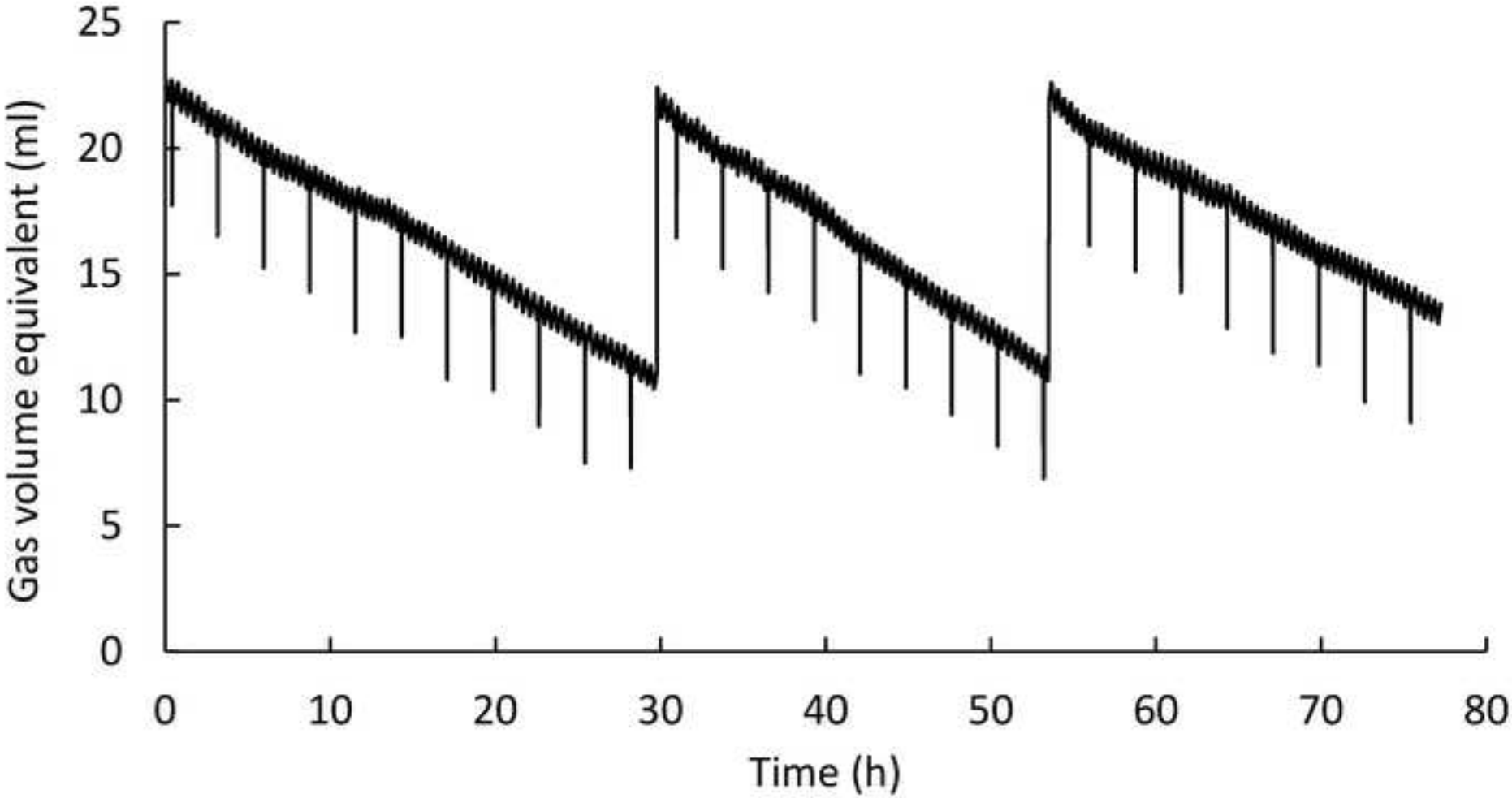

Gas volume equivalent (ml)
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Time (h)

Fig. 3

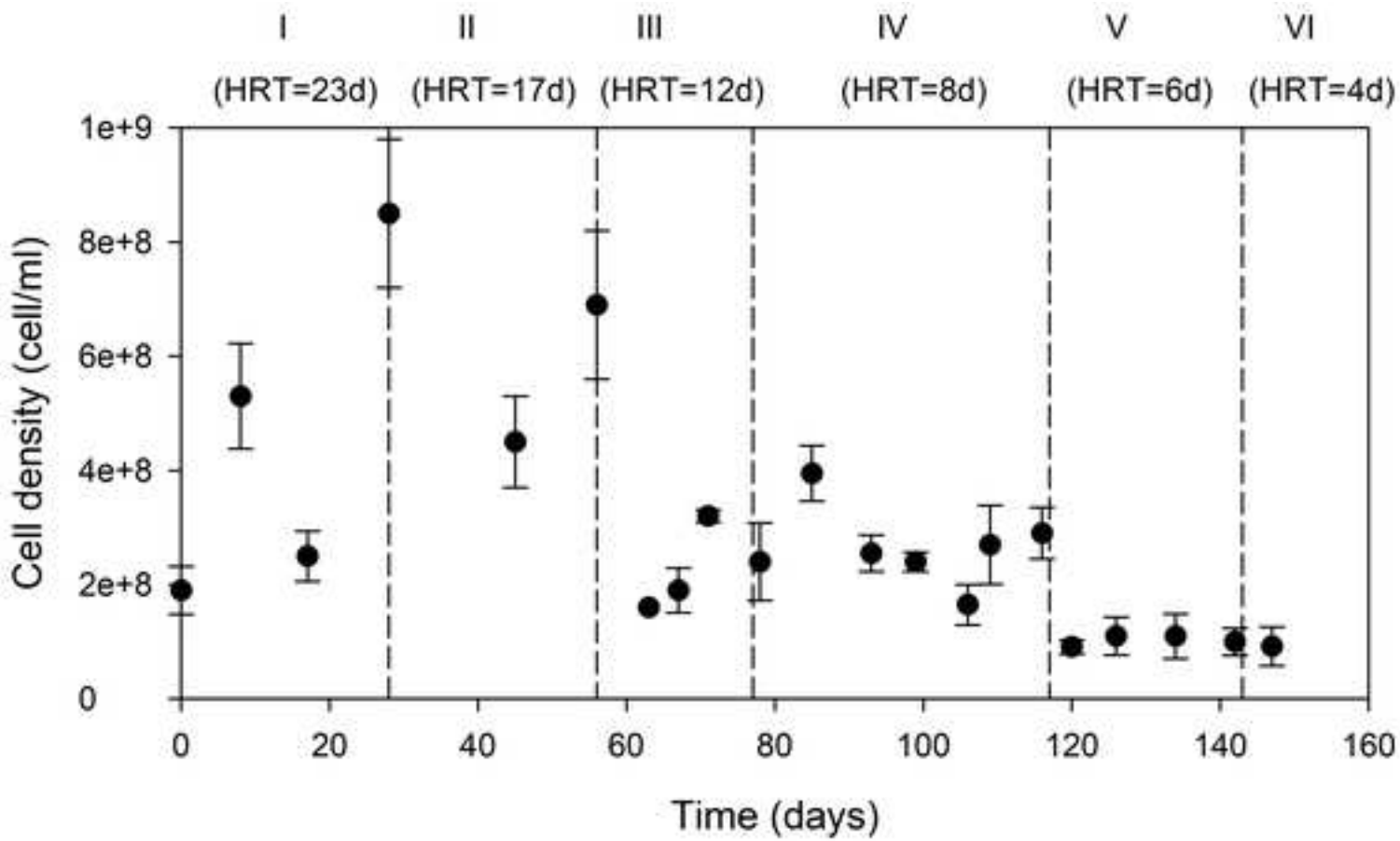

I
(HRT=23d)
II
(HRT=17d)
III
(HRT=12d)
IV
(HRT=8d)
V
(HRT=6d)
VI
(HRT=4d)
Cell density (cell/ml)
1e+9
8e+8
6e+8
4e+8
2e+8
0
0
20
40
60
80
100
120
140
160
Time (days)

Fig. 4

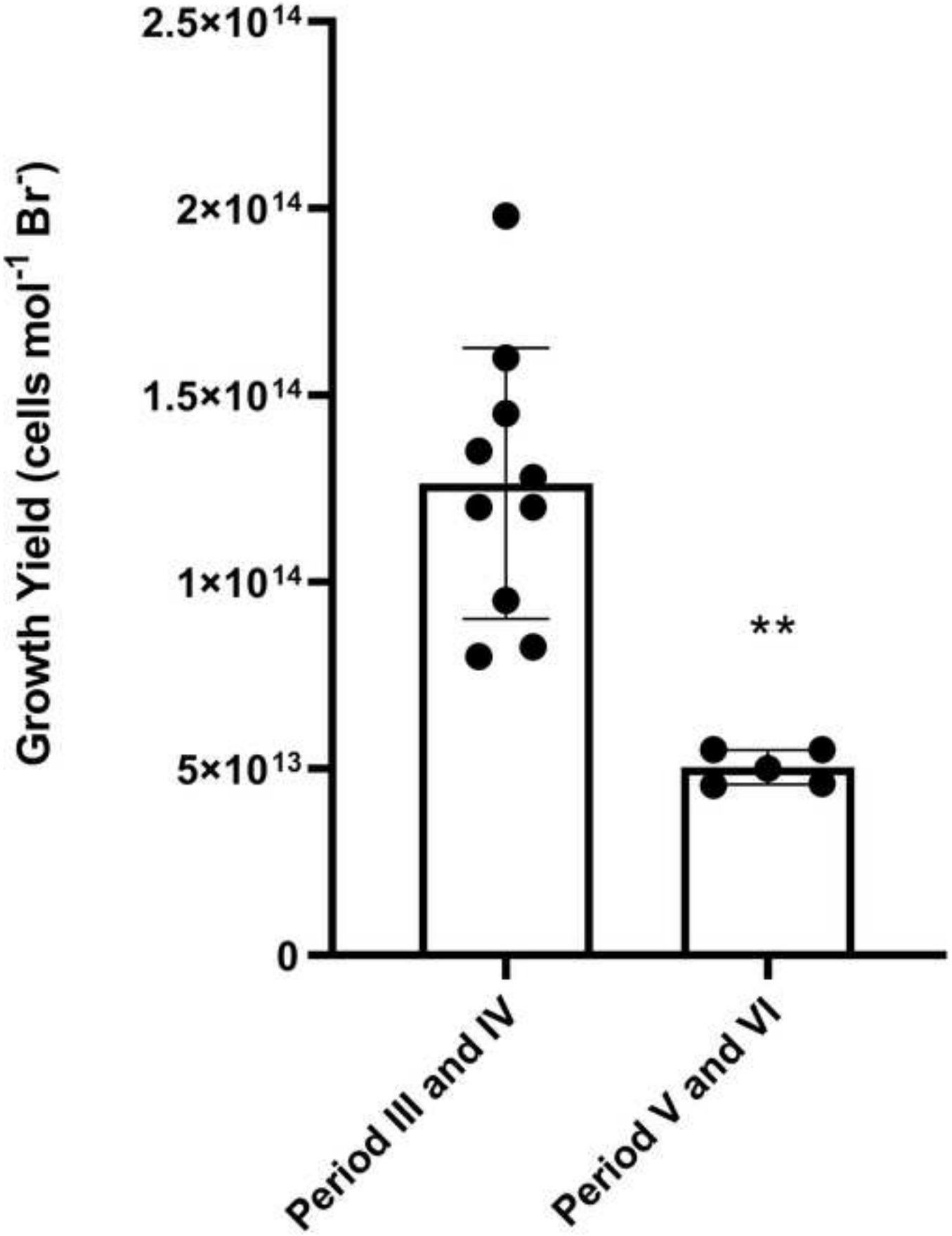
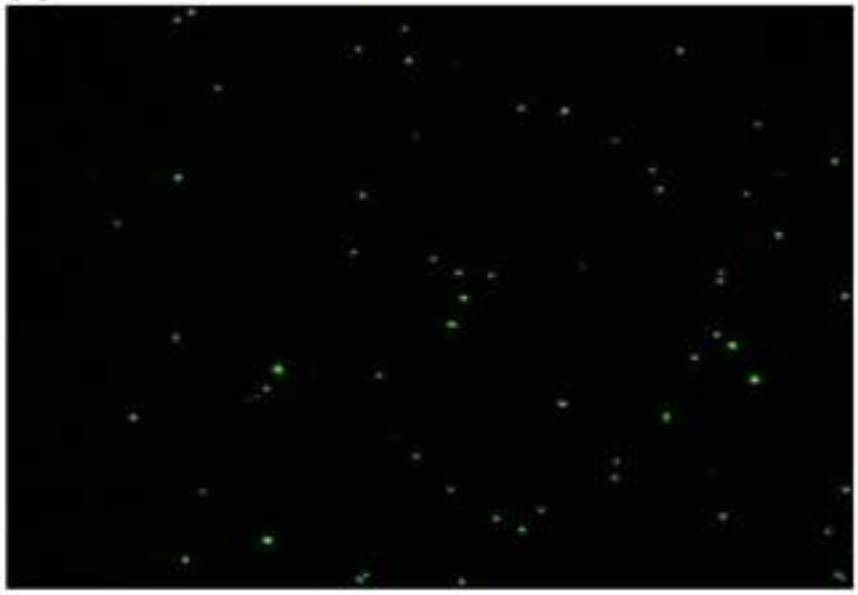
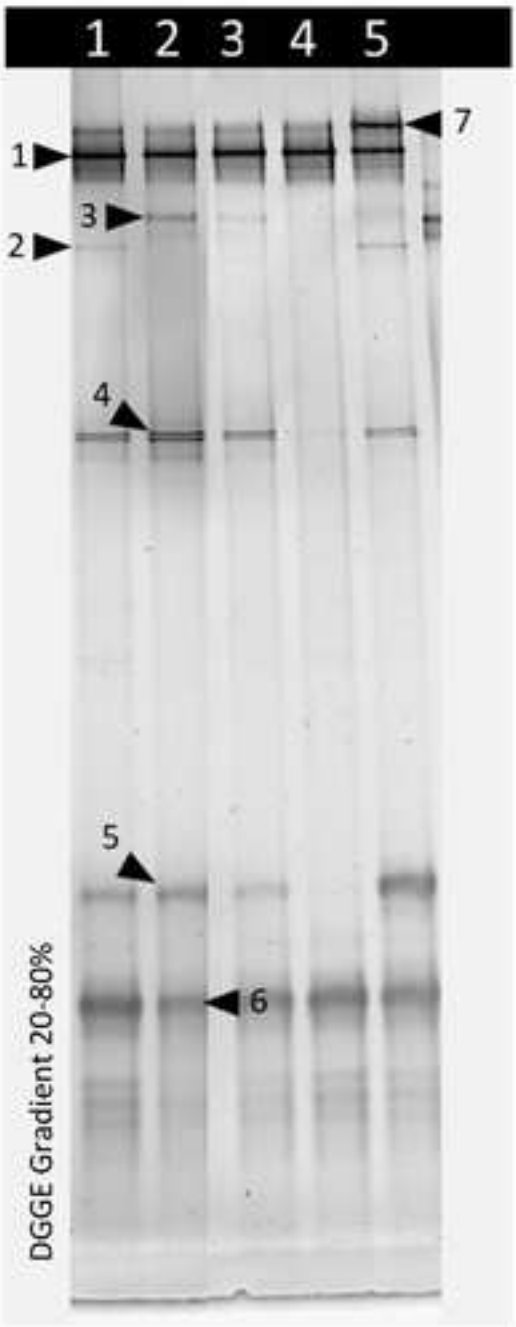

Growth Yield (cells mol-1 Br-)
2.5×10^14
2×10^14
1.5×10^14
1×10^14
5×10^13
0
**
Period III and IV
Period V and VI

Fig. 5





C

Band	Closest relative	Accesion no.
1	*Dehalococcoides* sp.	MN483297.1
2	*Dehalococcoides* sp.	MN483297.1
3	No significant match*	-
4	Uncultured *Desulfovibrionales* bacterium	DQ189060
5	Uncultured *Desulfovibrionales* bacterium	DQ189060
6	No significant match*	-
7	No results**	-

Table 1 Calculation of consumption of D-3,5-dibromotyrosine (D-DBT) and hydrogen in the headspace based on the dataset shown in Figure 3.

Calculation of D-DBT consumption		Calculation of hydrogen consumption	
D-DBT concentration in medium	5 mM	Gas consumed based on pressure change	31.3 mL
Medium added	140 mL (28 cycle × 5 mL per cycle)	Compensation due to pH change (approximate)	5.7 mL (pH dropped from 6.8 to 6.6, therefore CO_2% increased from 7.4% to 9.9%, headspace volume 230 mL)
Total DBT added	0.70 mmol	Total H_2 consumed	37.0 mL (at 30 ºC), or 1.49 mmol
Theoretical H_2 needed	1.40 mmol		

Table 2 Relative abundance (%) for the reductive dehalogenases (RdhA) identified by nLC-MS/MS analysis in strain CBDB1 culture fed with different halogenated compounds from previous works and from the work of the current study with dibromotyrosines. The pre-reactor was amended with D-3,5-dibromotyrosine (D-DBT), while the other 3,5-dibromtoryrosine samples correspond to the reactor fed with L-3,5-dibromotyrosine (L-DBT).

	Chloro- and bromobenzenes								3,5-dibromotyrosine						Other phenolic compounds					
RdhA	TeBB	TeBB	TeBB (1)	HBB (2)	HCB (1)	HCB_1st	HCB_2nd	HCB_3rd	Pre-	Day 8	Day 21	Day 57	Day 112	Day 144	TBBPA	BPB (2)	BPB_1st	BPB_2nd	BPB_3rd	BPB_4th
CbdbA80	0.2			*	*		3.8	13					0.1		*	*				
CbdbA84	0.2	6.1	*	*	*		1.5	1.7	0	0	0.1	0	4.3	0.3	*		0.2			
CbdbA88					*															
CbdbA238									0.9	0.7	1.8	4.6	1.6	0.8						
CbdbA1092									2.6	2.7	0.9	0.4	11	15	*	*	0.1	0.1	0.5	0.2
CbdbA1453			*	*	*		0								*					
CbdbA1455				*	*				0	0.1		0			*	*	0			
CbdbA1503															*	*				
CbdbA1588			*		*		0													
CbdbA1595									0		0.1									
CbdbA1598							0.2													
CbdbA1618			*	*	*				0.1	0	0.1	0		0.3	*	*				
CbdbA1624											0.1	0		0.1						
CbdbA1638					*				0.1	0.1	0.1	0.1		0.2						

Note:

(1) Seidel et al. 2018 Frontiers Microbiol

(2) Yang et al. 2015 ES&T

* indicating presence of an RdhA at non-quantifiable abundance